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Abstract: DNA repair deficiencies and genome instability are common features and hallmarks of cancer and are ubiquitously found in the full spectrum of malignant diseases. Heritable DNA repair deficiencies, for example, due to BRCA1 and BRCA2 mutations, and subsequent loss of heterozygosity in mammary, ovarian, and prostate carcinoma, are risk factors for the early development of cancer. Despite their detrimental role in tumorigenesis, these deficiencies also provide novel opportunities for treatment options. Current and future pharmacologic approaches in medical oncology rely on the exploitation of such genetically defined, tumor-specific Achilles' heels and integrate the genetic background of a tumor into the treatment strategy. For example, homologous recombination-corrupted, BRCA1/2-mutated tumors are becoming hypersensitive to inhibitors of an additional DNA-damage-repair mechanism and are successfully treated with respective molecular targeting agents such as PARP1 inhibitors. Patient stratification in radiation oncology today is primarily based on clinical parameters and uses highly sophisticated diagnostic imaging for treatment planning on the individual level. Radiation oncology only minimally takes the genetic makeup of tumors into account, and little attention has been given to the fact that the different modalities of ionizing radiation, such as photon and proton irradiation, may also induce differential damages and biological processes, which might again be influenced by the genetic makeup and mutational status of the tumor. However, radiation oncology is nowadays challenged to understand subtle differences induced by the different qualities of ionizing radiation, and to efficiently exploit and to integrate these differential responses in a personalized treatment approach alone and as part of combined treatment modalities with pharmacologic agents. Here we will review recent insights on the differential DNA damage responses to photon and proton irradiation and discuss their implications for combined treatment modalities with chemotherapeutic agents and small molecular targeting compounds.

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Combined Treatment Modalities for High-Energy Proton Irradiation: Exploiting Specific DNA Repair Dependencies

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Abstract

DNA repair deficiencies and genome instability are common features and hallmarks of cancer and are ubiquitously found in the full spectrum of malignant diseases. Heritable DNA repair deficiencies, for example, due to *BRCA1* and *BRCA2* mutations, and subsequent loss of heterozygosity in mammary, ovarian, and prostate carcinoma, are risk factors for the early development of cancer. Despite their detrimental role in tumorigenesis, these deficiencies also provide novel opportunities for treatment options. Current and future pharmacologic approaches in medical oncology rely on the exploitation of such genetically defined, tumor-specific Achilles' heels and integrate the genetic background of a tumor into the treatment strategy. For example, homologous recombination-corrupted, *BRCA1/2*-mutated tumors are becoming hypersensitive to inhibitors of an additional DNA-damage-repair mechanism and are successfully treated with respective molecular targeting agents such as PARP1 inhibitors.

Patient stratification in radiation oncology today is primarily based on clinical parameters and uses highly sophisticated diagnostic imaging for treatment planning on the individual level. Radiation oncology only minimally takes the genetic makeup of tumors into account, and little attention has been given to the fact that the different modalities of ionizing radiation, such as photon and proton irradiation, may also induce differential damages and biological processes, which might again be influenced by the genetic makeup and mutational status of the tumor. However, radiation oncology is nowadays challenged to understand subtle differences induced by the different qualities of ionizing radiation, and to efficiently exploit and to integrate these differential responses in a personalized treatment approach alone and as part of combined treatment modalities with pharmacologic agents. Here we will review recent insights on the differential DNA damage responses to photon and proton irradiation and discuss their implications for combined treatment modalities with chemotherapeutic agents and small molecular targeting compounds.

Keywords: proton irradiation; combined treatment modality DNA damage response; homologous recombination

Introduction

Approximately 50% of all cancer patients have an indication for radiation therapy at least once during the course of their disease, with an absolute number of patients steadily increasing assuming overall cancer rates remain unchanged ([Borras et al [1] and

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references therein). The main pillar of radiotherapy is photon irradiation with linear accelerators as source of ionizing radiation. Nevertheless, charged-particle-based approaches such as proton or carbon beams are stepping out of their niche and are becoming a reasonable alternative [2–4]. The high-dose deposition at the distal end followed by a steep decline as well as minimal lateral scattering allows superior tissue sparing and a dose reduction outside the planned margin [5]. The reduced volume of healthy tissue exposed to intermediate and low doses suggests a reduced risk of secondary malignancies accompanied by a reduced co-irradiation of dose-limiting organs-at-risk such as brain stem, spinal cord, oral cavity, or the optic nerve [6, 7]. Hence, current treatment decisions for particle beam therapy are primarily based on vulnerable tumor locations near organs-at-risk, and treated entities comprise uveal melanoma, skull-based and intracranial tumors, prostate and head and neck tumors [8, 9]. Moreover, the most common solid tumors in pediatric patients arise in the central nervous system [10] and a long life expectancy after treatment becomes aggravated by minimal safety margins in a delicate developmental stage. Since pediatric patients are prone to impaired brain development as well as progressive cognitive decline after conventional cranial radiation therapy, particle beam treatment emerges as a beneficial tool to reduce side effects in adult and especially in pediatric patients [11, 12].

Proton radiation therapy is characterized by its advantageous dose deposition and physics attributes, thereby taking primarily clinical aspects for treatment stratification into consideration. Preclinical experiments revealed an enhanced efficacy for proton versus photon irradiation. However, the cause for this increased efficacy is insufficiently known and differential biological stress responses induced by photon-based and particle-based irradiation are minimally investigated so far [13]. Differential “biologies” induced by the different sources of ionizing radiation may become relevant for the determination of optimal therapeutic strategies and be important for the concept of personalized medicine. It may allow adapting the therapy of an individual patient situation, taking both clinical and physical but also biological parameters into account.

Here we will provide an overview on recent preclinical insights on the differential DNA damage responses to photon and proton irradiation and their implications for combined treatment modalities with chemotherapeutic agents and small molecular targeting compounds.

Differential Biological Responses to Photon and Proton Irradiation

Intense preclinical in vitro and in vivo studies comparing photon and proton irradiation have illustrated differing relative biological effectiveness (RBEs) in dependence of endpoint, tissue, and positioning in the spread-out Bragg peak (SOBP) [14–17]. RBE deviations result from and clearly point to differential physicochemical and subsequent differential responses on the molecular and cellular level to photon versus proton irradiation. For example, faster production of reactive oxygen species [18] and enhanced levels of cell apoptosis [19] were identified after irradiation with low linear energy transfer (LET) proton irradiation in comparison to photon irradiation. Furthermore, differential gene expression in response to the 2 modalities of irradiation and increasing RBEs of cell killing along the axis of an SOBP also suggest different mechanisms being induced by photon and proton irradiation, which could be exploited as part of a combined treatment modality [20, 21]. These preclinical insights contrast with the generic constant RBE of 1.1 still applied in clinical practice [13, 22].

Exploitation of Differential DNA Repair Dependences

Genetically defined Chinese hamster ovary (CHO) cell lines with defects in different DNA repair systems were originally used to identify and characterize DNA double-strand break (DSB) machineries relevant for the repair of photon irradiation-induced DNA damage. These cell lines are interesting tools to characterize differential DNA damage responses and dependencies for specific DNA repair machineries in response to different qualities of ionizing radiation [23–25].

Cells have evolved 2 major DSB repair pathways, namely, the more error-prone nonhomologous end joining (NHEJ) process and homologous recombination repair (HRR) [26]. NHEJ is active throughout the cell cycle and is responsible for the repair of most ionizing radiation-induced DSBs in eukaryotic cells. Although very efficient in a quantitative way, the quality of repair by NHEJ can steadily decrease with increasing amounts of DNA damage [27].

Double-strand break repair by NHEJ relies on the initial binding of the Ku70/80 heterodimer, which results in the recruitment of DNA-dependent protein kinase, catalytic subunit, also known as DNA-PKcs. If required, specialized DNA nucleases and polymerases process the “dirty” ends before relegation of the broken strands by XRCC4–DNA ligase IV complex. HRR is initiated through the recognition of DSBs by the MRN complex followed by MRN- and CtIP-dependent 3′-5′ DNA resection [28]. The resulting single-stranded DNA (ssDNA) overhangs are first stabilized by the heterotrimeric ssDNA-binding complex RPA, which is later on replaced by Rad51 with the help of BRCA2. Rad51 nucleoprotein filaments then search for homology

and form Holliday junctions when complementary sequences are found. After ideally error-free rewriting of the damaged DNA site by DNA polymerases, these structures get resolved by several Holliday junction processing factors, including Rad51C-XRCC3 complex [29–31].

Detection and quantification of ionizing radiation–induced and subsequently repaired DNA DSBs represent major challenges in experimental radiobiology. Ideally, several complementary approaches should be combined that include both direct determination of DNA damage at the molecular level, including chromosomal aberrations, and indirect DNA damage–related signaling readouts, which are more easily detectable, such as γ H2AX foci. Phosphorylation of the histone variant H2AX has become a powerful tool to monitor DNA DSBs induced by ionizing radiation and was named γ H2AX because it was first observed in cells exposed to γ -rays [32]. Large numbers of γ H2AX molecules form at the site of DNA breaks and create bright foci that allow detection of individual DSBs. Thereby γ H2AX foci can be easily counted by using specific antibodies. γ H2AX foci counting has become a major experimental readout in basic and translational radiobiology to probe induction and repair of DNA DSBs at different time points following irradiation. Nevertheless, it only remains an indirect biomarker for DNA DSBs [33].

Using this set of CHO cells with defects in either the NHEJ (XR-C1; DNA-PKcs–deficient CHO cells) or HRR machinery (XRCC3–/–), our own group recently demonstrated differential DNA repair pathway choices in response to photon and proton irradiation. To avoid additional influences due to increasing LETs across the SOBP, cells were always irradiated in the middle of an SOBP with a length of 5 cm and a maximum proton energy of 138 MeV (Center for Proton Therapy at the Paul Scherrer Institute, PSI-Villigen, Switzerland) [34]. The formation of so-called γ H2AX foci at the site of DSBs was used to quantify irradiation-induced DSBs. No significant differences in the number of DNA DSBs induced by the 2 types of irradiation could be detected; however, the repair kinetics in XRCC3-lacking cells were clearly delayed after proton irradiation with elevated numbers of residual γ H2AX foci after irradiation. Additionally, the HRR-defective cells proved to be markedly hypersensitive to proton irradiation, resulting in an increased RBE in comparison to the RBE determined in wild-type cells. The RBE (survival fraction as endpoint) increased from RBE_{37%}: 1.25 ± 0.05 and RBE_{10%}: 1.29 ± 0.04 in the wild-type cells to RBE_{37%}: 1.54 ± 0.10 and RBE_{10%}: 1.44 ± 0.06 in the HRR-deficient cells. While DNA-PKcs–defective cells were overall more sensitive to both types of ionizing radiation than wild-type cells, defective DNA-PKcs did not result in hypersensitivity towards proton irradiation. Even though these quantitative readouts were only correlated with the γ H2AX-foci assay, these results indicate a differential quality of DNA damage by proton versus photon irradiation with a specific requirement for HRR for efficient DNA repair and enhanced cell survival in response to low LET proton irradiation. Interestingly, differential patterns of chromosomal aberrations were also identified in response to the 2 types of ionizing radiation and in dependence of the HRR status. Of note, lack of NHEJ activity in genetically defined knockout mouse embryo fibroblasts was shown to play the more important role for cell survival in response to high LET irradiation than to photon irradiation and thus, targeting of NHEJ might preferentially sensitize to high LET irradiation [35]. A differential response and involvement of the 2 major DNA DSB machineries in dependence of high versus low LET radiation might be further affected by the mutational status of the targeted cells and additional DNA DSB repair backup mechanisms, such as alternative or B-NHEJ [36].

HRR dependence for proton irradiation–induced DNA damage was also tested in established cancer cell lines. Interestingly and in contrast to their wild-type counterpart cell line, ovarian carcinoma cells lacking intact BRCA2 expression were also hypersensitive towards proton in comparison to photon irradiation, which corroborated this hypothesis originally tested in the CHO-based model cell system. Likewise, A549 non-small cell lung cancer cells depleted of the HRR essential protein Rad51 were clearly hypersensitive to proton irradiation in comparison to control cells [37]. Rad51 downregulation also induced a tremendous delay in γ H2AX-foci repair kinetics. As such, a preference of proton-induced DNA damage towards HRR might become relevant for clinical stratification of patients carrying mutations in this DNA repair pathway.

Combined Treatment Modalities with HRR-Interfering Agents

Likewise, a state of corrupted HRR activity and thus enhanced sensitivity towards proton irradiation could be achieved by a combined treatment modality with specific inhibitors of HRR. Direct pharmacologic targeting of the HRR machinery has proven to be largely unsuccessful in the past years. However, several chemotherapeutic agents exist, which eventually downregulate Rad51 protein levels—including the broad-range clinically relevant histone deacetylase inhibitor SAHA (Vorinostat) [38]—and thereby also reduce HRR. Low dose exposure to SAHA for 24 hours was sufficient to reduce Rad51 levels down to approximately 10% of the basal level, without affecting critical elements of NHEJ such as Ku80 or DNA-PKcs protein levels. Similar to the results obtained in the tumor cells with a corrupted HRR machinery, DNA repair was also strongly delayed in

SAHA-pretreated cells after proton irradiation, but only minimally after photon irradiation, corroborating this HRR-oriented mechanism of action of SAHA.

Recently, Gerelchuluun et al [39] investigated SAHA-based radiosensitization in lung carcinoma and normal human fibroblasts irradiated with photon, low LET proton, and carbon ion-based irradiation. SAHA sensitized to low LET radiation to a greater extent than to carbon ion-based irradiation and even more so to proton irradiation at low SAHA concentrations. Even though they could not directly link their results to HRR, SAHA also delayed DNA repair kinetics. More important, SAHA-induced radiosensitization was not prominent in normal fibroblasts, which suggests a selective cancer cell-related mechanism, of interest towards an enhanced therapeutic window.

Complementary experiments were performed with pharmacologic agents selectively inhibiting NHEJ. Interestingly, the DNA-PKCs inhibitor NU7026 sensitized lung carcinoma and glioblastoma cells in response to both types of irradiation, yet to a higher extent for photon irradiation, with a dose modifying factor at 10% survival (DMF_{10}) of 1.91 ± 0.05 , than for proton radiation (1.49 ± 0.06) for lung and with a DMF_{10} of 1.49 ± 0.02 versus 1.2 ± 0.11 for the glioblastoma cells. Strikingly, NU7026 treatment only minimally interfered with repair of proton-induced DNA damage, while more than half of γ H2AX foci remained unrepaired after 24 hours in the photon-irradiated cells [37]. Thus, while inhibitors of NHEJ more specifically sensitize to photon irradiation, pharmacologic agents that directly downregulate the DNA repair capacity of HRR might more specifically sensitize to proton irradiation.

An RBE-oriented lung cancer cell line screen performed by Liu et al [40] (research group of Henning Willers at Massachusetts General Hospital, Boston) also linked defects in a specific DNA-damage-repair machinery to hypersensitivity to proton irradiation [40]. A selection of NSCLC cell lines was irradiated in the middle of an SOBP, and in 3 of 17 cell lines an increased RBE could be linked to alterations of the Fanconi anemia (FA)/BRCA pathway of DNA repair, which is part of replication-coupled HRR [40]. Control experiments performed in wild-type and FANCD2-depleted but otherwise isogenic lung cancer cells validated the relevance of this pathway and also supported the hypothesis of specific proton hypersensitivity due to HRR defects. Furthermore, the role of FA/BRCA pathway in hypersensitization against proton irradiation was further backed up by the same group focusing on 2 additional downstream elements of this pathway, namely, Slx4(FancP) and Mus81 [41]. Slx4- and Mus81-deficient cells also demonstrated enhanced sensitivity towards proton irradiation as compared to their isogenic wild-type counterpart cells. As such, genomic profiling of DNA damage repair-associated genes might become relevant in the future for clinical stratification of patients and be part of personalized biology-adapted treatment regimens.

DNA damage can be traced in vitro and ex vivo by the generation of so-called foci, which represent specific protein aggregates at the site of DSBs. Interestingly, these studies also revealed that proton irradiation- and photon irradiation-induced foci exhibit a different size, in particular in dependence of the HRR status. As suggested, DNA repair-associated foci could therefore be used as functional biomarker to identify repair-defective and proton irradiation hypersensitive tumors [40, 42]. It will be of interest to determine the phenotype of proton-induced foci in genetically HRR intact cancer cells but cotreated with HRR-directed pharmaceutical inhibitors. A large amount of data and calculations on DNA damage in response to particle irradiation exist, which are derived from different sources of ionizing radiation and which have been incorporated into relevant modeling studies [43, 44]. However, we have only limited experimental data on differential DNA damage, which were obtained in parallel in the same genetically defined cell systems with specific DNA damage repair deficiencies, and which were induced by clinically relevant proton and photon irradiation. The initial amount of DSBs per physical dose of proton irradiation is similar to the number of lesions caused by the same dose of photon irradiation, based on the initial formation of γ H2AX foci [34, 37]. However, proton irradiation might cause slightly more complex clustered DNA damage, which is most probably due to the slightly increased LET values even in the middle of the SOBP. While DNA DSB-proficient cells repair these damages equally well, FA/BRCA pathway- and thus replication-coupled HRR-corrupted cells encounter greater difficulties in repairing replication forks that collide with clustered proton damage. Eventually, impaired repair in these mutated tumor cells will translate into a hypersensitivity towards proton irradiation. As such, the genetic status could make tumors more susceptible to proton radiation therapy and could contribute to the definition of a variable instead of a generic RBE [45].

Similar to SAHA, other clinically relevant pharmaceutical agents and compounds in early developmental stage exist, which directly or indirectly reduce HRR activity and have been tested in combination with photon irradiation. For example, Gleevec (Novartis Pharma, Switzerland), which is used to treat chronic myelogenous leukemia, gastrointestinal stromal tumors, and a number of other malignancies, also reduces RAD51 and sensitizes for ionizing radiation [46]. Likewise, inhibitors of the hepatocyte growth factor receptor MET (mesenchymal-epithelial transition), which is overexpressed in numerous types of human tumors and considered a prime target in clinical oncology, sensitize for ionizing radiation via

reduced formation of the RAD51-BRCA2 complex [47]. Even classic chemotherapeutic agents like gemcitabine, which are clinically applied in combination with radiation therapy, sensitize via selective targeting of HRR [48]. As such, treatment combinations with agents cotargeting HRR might require a reduced dose of proton radiation to achieve the same treatment outcome as with photon irradiation. On the other hand, agents targeting NHEJ might be less effective in combination with proton irradiation. Most combined treatment modalities have been investigated with photon irradiation. It will be now important to qualitatively as well as quantitatively determine the differential outcome combining these agents also with clinically relevant proton irradiation.

ADDITIONAL INFORMATION AND DECLARATIONS

Conflicts of Interest: The authors have no conflicts of interest to disclose.

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